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DNA and Total Protamine Masses in Individual Sperm From Fertile Mammalian Subjects

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The total amount of phosphorus and sulfur inside the nuclei of individual bull, stallion, hamster, human, and mouse sperm from fertile subjects has been measured using Particle Induced X-ray Emission (PIXE). Using the sulfur masses, we determined the total protamine (protamine 1 plus protamine 2) mass within the sperm nuclei of each species. Using the phosphorus masses, we determined the DNA mass present within the sperm nuclei of each species. The results reveal that although the relative proportion of protamine 1 to protamine 2 varies among the species examined, the total protamine mass to DNA mass ratio is similar in bull, stallion, hamster, and mouse sperm nuclei. In contrast, mature human sperm nuclei were found to contain significantly less protamine. This observation is consistent with other studies, which suggest that as much as 15% of the DNA in human sperm remain packaged by histones. Using the data obtained for bull sperm, the length of DNA that could be covered by each protamine 1 molecule in bull sperm has been estimated. Making the assumption that the size of the protamine 1 binding site on DNA is similar in the sperm of these species, the length of DNA covered by a single protamine 2 molecule also has been estimated. © 1996 Wiley-Liss, Inc.

Key terms: Sperm, protamine 1, protamine 2, DNA, Particle Induced X-ray Emission (PIXE), electrophoresis, microdensitometry, histones, binding site

During spermiogenesis in mammals, the structure of spermatid chromatin is completely reorganized and the DNA is repackaged by small proteins called protamines. Two different types or families of protamine molecules—protamine 1 and protamine 2—participate in this process. The amino acid sequences of protamine 2 molecules differ from protamine1 sequences in several ways. Protamine 2 is substantially larger than protamine 1 and usually has a high content of histidine. Protamine 1 rarely contains histidine. A further difference is that the clusters of arginine residues in protamine 2 appear to be more evenly distributed throughout the length of the molecule.

Studies have shown that the mature sperm of bull (16,26), ram (24,33), boar (37,37), rat (22), and guinea pig (14) contain only protamine 1. However, both protamine 1 and 2 are known to be present in mouse (8,13, 29), hamster (17), stallion (2,10), human (7,30,35), and other primate sperm. The sperm of stallions (28), humans (7,19,30,35), and certain primates also contain a third protamine, a variant of protamine 2 that has been designated protamine 2b. The amino acid sequence of protamine 2b has been found to be nearly identical to that of protamine 2a (the larger form of the two protamines is designated 2a when both proteins are present in sperm). For example, in human sperm protamine 2b lacks only the first three amino-terminal amino acids, arg-thr-his, of protamine 2a (3,7,19,27).

The relative proportions of protamine 1 and protamine 2 molecules present in mature sperm have been observed to differ widely among species. For instance, mouse sperm contain twice as much protamine 2 as protamine 1, and hamster sperm contain twice as much protamine 1 as protamine 2. Whereas the sperm of many species contain only protamine 1, no species has been found that uses only protamine 2 to package its DNA. These findings

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indicate that protamine 1 and protamine 2 molecules cannot interact with a particular or universal stoichiometry to package DNA in sperm chromatin.

Biochemical studies of sperm in normal and infertile human males have also provided evidence that protamine 2 may play a special role in sperm chromatin organization and fertility in humans. In every case but one in a study of infertile males, the proportion of protamine 2 was markedly reduced in sperm chromatin [9]. However, the most convincing evidence that protamine 2 may perform some unique function in sperm chromatin involves its synthesis and metabolism. Protamine 2 is formed as a precursor that is deposited onto DNA and subsequently processed by a series of selective proteolytic cleavages. Protamine 1 is not synthesized as a precursor. Nevertheless, neither of these observations provide conclusive evidence that protamine 2 performs a unique function. If we are to understand the importance of protamine 2 to male fertility, we must first determine how protamine 1 and 2 interact to package sperm DNA. Remarkably little is known about this process. In this study we measured the total amount of phosphorus and sulfur inside the nuclei of individual mature bull, stallion, hamster, human, and mouse sperm from fertile subjects using Particle Induced X-ray Emission (PIXE). Using the sulfur masses, we determined the total protamine (protamine 1 plus protamine 2) mass within the sperm nuclei of each species. Using the phosphorus masses, we determined the DNA mass present within the sperm nuclei of each species. The protamine 2 to total protamine ratio has been determined to vary widely between species, from 0 for bull sperm through 0.14 for stallion sperm, 0.34 for hamster sperm, 0.43 for human sperm, to 0.67 for mouse sperm (6,8,9,17,29). The present studies reveal that although the relative proportion of protamine 1 to protamine 2 is highly variable, the total protamine mass to DNA mass ratio appears to be similar in bull, stallion, hamster, and mouse sperm nuclei. In contrast, mature human sperm nuclei were found to contain significantly less protamine. This observation is consistent with other studies, which suggest that as much as 15% of the DNA in human sperm remain packaged by histones (18,35). Using the data obtained for bull sperm (bull sperm contain only protamine 1), we estimated the length of DNA that could be covered by a single protamine 1 molecule. By making the assumption that the protamine 1 binding site on DNA does not change among species, we also have been able to use the present data to estimate the length of DNA covered by a single protamine 2 molecule.

MATERIALS AND METHODS

Mouse (*Mus musculus*) sperm were obtained from the epididymides of male retired breeders (FVB strain). The sperm used in these studies were pooled from three mice. Syrian hamster (*Mesocricetus auratus*) epididymal sperm were obtained from a single retired breeder. In both cases, the animals were sacrificed by cervical dislocation following anesthesia and the caudal portions of the epididymides were removed. Semen was released from the vas into 5 ml 0.15 M ammonium acetate by applying pressure to each epididymis. Bull (*Bos taurus*) sperm were obtained from pooled samples of semen (American Breeders Service, De Forest, WI) collected from fertile males. Stallion (*Equus caballus*) semen was collected by Dr. Charles Love (New Bolton Center, University of Pennsylvania, Philadelphia) from stallions of demonstrated fertility. Human semen samples were obtained from a fertile volunteer. Aliquots of semen from each species were diluted with 0.15 M ammonium acetate (2 ml) to wash the sperm. The samples were centrifuged (4124 × g for 5 min) to pellet the sperm cells and resuspended in 0.15 M ammonium acetate (2 ml).

Determination of Phosphorus and Sulfur Masses in Sperm Nuclei

Initial microbeam PIXE spatial maps of individual freeze-dried whole sperm (i.e., intact sperm with nuclear membranes and tails) indicated that some sulfur was contained in the tails and possibly the acrosome/nuclear membranes. To obtain accurate protamine and DNA values, these measurements indicated that it would be preferable to examine amembraneous sperm nuclei free of contaminating nuclear membranes and tails. For each species, pure sperm nuclei free of contaminating nuclear membranes and tails were obtained by treating the washed sperm with dithiothreitol (DTT) and mixed alkyl trimethylammoniumbromide (MTAB) as previously described (8). The suspension was centrifuged at 4124 × g for 3 min selectively to pellet the sperm chromatin (amembraneous nuclei), which was subsequently washed and re-suspended twice in doubly distilled deionized water to remove any remaining DTT and MTAB. Following each resuspension in the doubly distilled deionized water, the solution was centrifuged at 4124 × g for 3 min selectively to pellet the amembraneous nuclei. Prior to the final centrifuging, the solution was sonicated for 15 s using a Teflon-coated probe to help reduce clumping of amembraneous nuclei. It has been demonstrated that treatment of fertile mammalian sperm by this process results in the complete removal and dissolution of the acrosome, nuclear membrane, and tail of the sperm, leaving the amembraneous nucleus intact (8). The characteristic shape of the sperm nucleus is also retained. These amembraneous nuclei may then be used for subsequent biochemical analyses avoiding possible contamination with nuclear membranes and tail fragments (8). Analyses of the MTAB supernatants by gel electrophoresis have shown that no basic nuclear proteins are extracted by this treatment (data not shown).

The sperm MTAB-treated nuclei were resuspended in a small volume of doubly distilled deionized water and ~ 5 μl of the suspension was micro-pipetted onto ultra-clean, 1-μm-thick, transparent nylon foils stretched over a 15 mm diameter hole in a plastic support frame. The nuclei were allowed to settle onto the nylon surface for 5 min and the excess buffer was removed with a Pasteur pipette and blotting paper. The nylon foils containing sperm were rapidly plunged in liquid nitrogen and subsequently

dried in a lyophilizer at a pressure of < 100 mTorr for at least 6 h. The freeze-dried samples were stored in a clean, dry environment prior to microprobe analysis. Optical studies revealed that the characteristic morphology of the nuclei was retained after lyophilization.

Amembraneous nuclei from individual sperm were examined with the nuclear microprobe facility located at the Lawrence Livermore National Laboratory (31). For each species only mature sperm nuclei that possessed the characteristic normal morphology, size, and shape were irradiated. Particle induced x-ray emission (PIXE) data were obtained using incident 3 MeV proton microbeams. PIXE is an accurate, absolute, fully quantitative technique that produces sample elemental concentrations from the x-ray yield induced by particle bombardment [21]. X-rays were detected with a Si(Li) detector that subtended a solid angle of ~100 msr. The detector was located at an angle of 135° with respect to the incident beam. Charge was collected in a biased Faraday cup located behind the sample. X-rays were recorded in list mode along with coincident beam spatial co-ordinates arising from scanning the beam electrostatically over the sample in a point by point raster mode. The use of PIXE to measure elemental concentrations inside sperm nuclei has been described in greater detail elsewhere (11).

For the measurements reported here, beam currents of up to 900 pA focused down to spot sizes of between 2–3 μm were repeatedly scanned over areas of ~23 × 22 μm^2. Within each pass, 0.1 nC was deposited on each beam location. Areas were irradiated with an exposure of up to 1.5 μC. Each irradiated area contained the amembraneous nucleus from a single sperm. Figure 1 shows spatial distributions of phosphorus (P) and sulfur (S) x-rays from the irradiation of a single bull sperm amembraneous nucleus with an exposure of 0.6 μC. These maps reveal that P and S are tightly localized in the center of the scan corresponding to the location of the amembraneous nucleus and background levels primarily arising from secondary electron bremsstrahlung are low. P and S background levels in each scanned area were below minimum detectable limits of ~ 1 ng/cm^2. P in DNA and S in protamine are tightly bound in sperm chromatin and are nonlabile; it is unlikely that these elements are lost from the amembraneous nucleus during sample preparation. Data were reduced off-line so that x-ray spectra corresponding to the nuclei could be extracted from each irradiated region (5). X-ray spectra were analyzed with the PIXEF spectrum fitting code (4). P and S counting statistics from each amembraneous nucleus examined were better than 3.5% and typically better than 2%. Figure 2 shows an x-ray spectrum from the amembraneous nucleus of a bull sperm. A series of thin film calibration standards were used to measure the efficiency of the x-ray detection system. To cross check our detector's x-ray detection efficiency two more thin film standards, one known to contain sulfur at 12.0 ± 0.6 $\mu g/cm^2$ and the other known to contain phosphorus at 15.3 ± 0.6 $\mu g/cm^2$ were analysed using the same incident beam and beam energy that were used to analyze the sperm. Our microbeam PIXE system yielded a measured sulfur thickness of 12.4 ± 0.5 $\mu g/cm^2$ for the sulfur bearing standard and a measured phosphorus thickness of 15.6 ± 0.5 $\mu g/cm^2$ for the phosphorus bearing standard. P and S masses (units of gram) inside the nuclei of individual sperm were calculated using the thin film approximation (15), the total scan area, and the exposure delivered to the total scan area. The measurement of P and S masses has a quantitative accuracy of < 7% (4% detector efficiency/charge collection and 5% scan area calibration/thin film approximation added in quadrature).

Isolation of Protamines and Analysis of Protamine Ratios

The sulfur mass measured via PIXE is used to derive the total protamine mass in an amembraneous nucleus. Stallion, hamster, human, and mouse sperm nuclei contain two types of protamine molecules, which differ in their sulfur (cysteine and methionine) contents. Consequently, conversion of the sulfur contents of these nuclei into a measure of the protamine content requires that we know the relative proportion of the protamines present in the nucleus. The proportion of the different forms of protamine present in bull, hamster, mouse, and human sperm nuclei already have been determined and reported (6,8,9,17,29). For stallion sperm, the ratio of protamine 1 to protamine 2 was determined in this study. This was achieved by dissolving MTAB- and DTT-treated amembraneous stallion sperm nuclei in 5 M guanidine chloride (GuCl). This solution was immediately diluted with a stock solution of urea, 2-mercaptoethanol and sodium chloride to a final concentration of 0.5 M GuCl, 3M urea, 0.5 M 2 mercaptoethanol, and 2 M sodium chloride as described previously for mouse sperm nuclei (8). After standing at 4°C for 60 min, the DNA was precipitated by the addition of HCl to 0.5 N, pelleted at 14,500 × g for 10 min and the protamines precipitated from the supernatant with TCA (final concentration of 20%). Following repeated washing in acidified acetone, the protamine pellet was dried in vacuo.

The electrophoresis of stallion protamine was performed in 10 cm acid-urea gels and the gels were stained with Naphthol Blue Black and destained as previously described (8). The gel bands were scanned with a Shimadzu Flying Spot microdensitometer (Shimadzu, Pleasanton, CA) and the areas under the protamine 1 and protamine 2 peaks were determined and used to calculate the protamine 2/total protamine content of the stallion sperm chromatin.

Conversion of Phosphorus and Sulfur Masses to DNA and Protamine Masses

From the P and S masses, the DNA and protamine content inside the nucleus of a single sperm can be derived. These DNA and protamine mass calculations assume that all the phosphorus is contained in DNA and all the sulfur is contained in the protamine within the amembraneous sperm nucleus. Bull, hamster, stallion, and mouse sperm chromatin primarily consists (>95%) of DNA and prot-

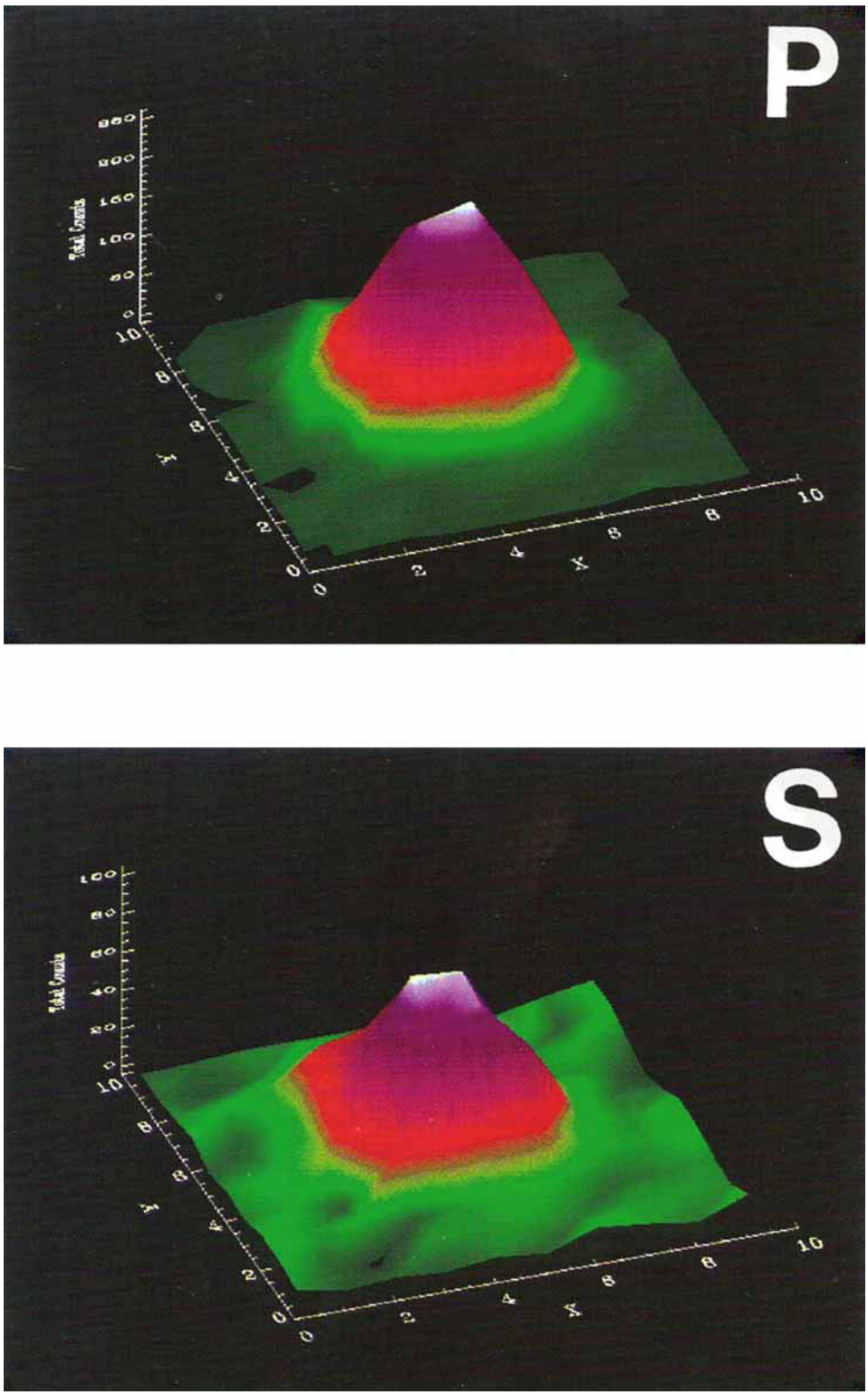

FIG. 1. Nuclear microscopy spatial maps of phosphorus (P) and sulfur (S) x-ray yields from the amembraneous nucleus of a single bull sperm. Data were collected with a 2.5 μm diameter incident beam spot size, and the scan size had dimensions of 23 × 22 μm^2. Each image consists of 10 × 10 pixels in the x and y dimensions. The z dimension in each image corresponds to number of x-ray counts and has been augmented with a color scale, ranging from black (zero x-ray counts), through green, orange, red, purple, to white (maximum x-ray counts). The peaked region in the center of each image corresponds to the amembraneous nucleus. In both the P and S images, the limits of the nucleus are approximately at the midgreen light-green color boundary where a sharp increase in the measured X-rays begins.

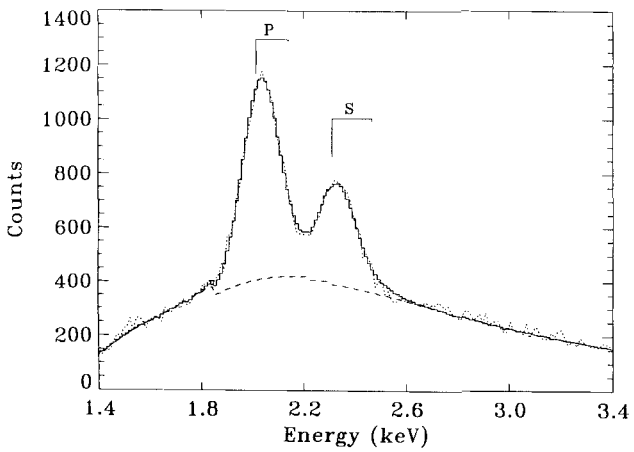

FIG. 2. PIXE spectrum between 1.4 and 3.4 keV from the amembraneous nucleus of a single bull sperm. The dotted line corresponds to the raw x-ray data. The dashed line is the computed estimate of the x-ray background, whereas the solid line shows the computed fit to the x-ray peaks using the PIXEF spectrum fitting code. The spectrum was formed from a dose of 0.24 μC to the nucleus. Phosphorus and sulfur x-ray lines are labeled.

amines (unpublished data). The phosphorus content of sperm chromatin provides an accurate measure of the DNA content for sperm do not contain RNA and protamines do not contain significant amounts of phosphorus. Further, although protamines can be phosphorylated, the overall phosphorylation of protamines in mature sperm is slight (25). A DNA base pair has a molecular weight of 660 g/Mol and contains two phosphorus atoms but no sulfur. The molecular weight of phosphorus is 30.97 g/Mol. To obtain the DNA mass in sperm chromatin the phosphorus mass is multiplied by (660/(2 × 30.97)).

DNA does not contain a significant amount of sulfur, whereas cysteine is the only sulfur containing amino acid in bull, stallion, hamster, and mouse protamine and human protamine 2. Human protamine 1 also contains sulfur in a single methionine residue. Because the protamines, which constitute the majority of the protein inside bull, stallion, hamster, and mouse sperm nuclei, contain a known number of cysteine and methionine residues per molecule, sulfur should be an effective measure of the total protamine content in these species. Nuclear lamins and matrix proteins are also present within sperm nuclei (38) and can potentially contribute to the measured sulfur content. However, in general, these proteins contain much less sulfur than protamines and only contribute a small fraction (< 10%) of the total mass of the amembraneous nuclei. Lamins and matrix proteins would contribute a few percent at most to the total sulfur mass in mature sperm nuclei.

The total protamine content can be determined by multiplying the sulfur mass by:

$$\sum_{i=1}^{K} \frac{M(P_i) \times R_i}{M(S) \times N(S_i)} \qquad (1)$$

where K is the number of forms of protamine, $M(P_i)$ is the molecular weight of protamine i, R_i is the ratio of number of molecules of protamine i to total protamine molecules in the chromatin M(S) is the molecular weight of sulfur, and $N(S_i)$ is the number of sulfur atoms in the i^{th} form of protamine. Although several studies suggest that human sperm chromatin contains a small amount of sperm specific histones (18,30,35), none of these histones contain sulfur. Consequently, the total protamine mass in human sperm was also calculated using equation 1 assuming all the sulfur signal arises from protamine. Table 1 lists the molecular weights and number of sulfur atoms per molecule for each form of protamine found in bull, stallion, hamster, human, and mouse sperm nuclei together with the relative amount of each form of protamine 2 to total protamine. The relative amounts of each form of protamine 2 to total protamine in hamster, human, and mouse sperm come from previously published values (6,8,9,17, 29). The relative amounts of each form of protamine 2 to total protamine in stallion sperm comes from the protamine 2 to total protamine value found in this study and the protamine 2a to protamine 2b ratio of ~0.10 determined by Pirhonen [28].

Using mouse sperm as an example illustrates the use of equation 1 to calculate protamine conversion factors. Mouse sperm contain both protamine 1 and protamine 2. The ratio of protamine 2 to total protamine in fertile mouse sperm is 0.67. Mouse protamine 1 has a molecular weight of 6827.3 g/Mol and contains nine sulfur atoms, whereas mouse protamine 2 has a molecular weight of 8594.7 g/Mol and contains seven sulfur atoms. Sulfur has a molecular weight of 32.06 g/Mol. To obtain the total amount of protamine in a fertile mouse sperm, the sulfur mass is multiplied by

$$\frac{8594.7 \times 0.67}{7 \times 32.06} + \frac{6827.3 \times 0.33}{9 \times 32.06} = 33.5$$

RESULTS

Nineteen bull, stallion, hamster, and human sperm and 20 mouse sperm were analyzed and the results are shown in Table 2. Using the mass of phosphorus measured for each cell to calculate its DNA content, the amembraneous nuclei of the five species of sperm cells were found to contain approximately the same amount of DNA (3.23–3.42 pg/cell). The differences in DNA content between species are small and may not prove to be significant if larger populations of cells are analysed.

Although the sulfur mass contained in the amembraneous nuclei of sperm from these five species of mammals were measurably different, the total mass of protamine present in the nucleus was found to be similar in bull, stallion, hamster, and mouse sperm (Table 2). This reflects a combination of factors, including known differences in the cysteine and methionine content of the protamines (amino acid sequences or cysteine and methionine content are known for protamine 1 and 2 present in the sperm of each species tested) and the relative pro-

Table 1
Protamine Molecular Weights and Number of Sulfur Atoms in Each Form of Protamine in the Bull, Stallion, Hamster, Mouse, and Human Sperm Used in This Study[a]

	Bull	Stallion	Hamster	Mouse	Human
Protamine 1 molecular weight (g/Mol)	6627.3	6702.4	6869.7	6827.3	6692.2
Sulfur atoms per protamine 1 molecule	7	8	9	9	7
Protamine 2 molecular weight (g/Mol)	NA	NA	8599.0	8594.7	NA
Protamine 2a molecular weight (g/Mol)	NA	8418.5	NA	NA	7653.4
Protamine 2b molecular weight (g/Mol)	NA	7980.0	NA	NA	7258.9
Sulfur atoms per protamine 2 molecule	NA	NA	7	7	NA
Sulfur atoms per protamine 2a molecule	NA	6	NA	NA	5
Sulfur atoms per protamine 2b molecule	NA	5	NA	NA	5
Protamine 2/total protamine	0	NA	0.34 ± 0.01	0.67 ± 0.01	NA
Protamine 2a/total protamine	0	0.124 ± 0.006	NA	NA	0.29 ± 0.02
Protamine 2b/total protamine	0	0.014 ± 0.001	NA	NA	0.14 ± 0.02

[a]Ratio of the number of each form of protamine 2 molecule to total number of protamine molecules for the amembraneous nuclei of each species is also listed.
NA = not applicable.

Table 2
Average Phosphorus and Sulfur Mass Measurements in Bull, Stallion, Hamster, Mouse, and Human Amembraneous Sperm Nuclei, Together With Number of Sperm Examined for Each Species[a]

	Bull	Stallion	Hamster	Mouse	Human
Number of sperm heads analyzed	19	19	19	20	19
Phosphorus mass (10^{-13} g)	3.21 ± 0.09	3.09 ± 0.09	3.14 ± 0.09	3.16 ± 0.08	3.04 ± 0.08
Sulfur mass (10^{-13} g)	0.98 ± 0.05	0.99 ± 0.05	1.04 ± 0.05	0.86 ± 0.05	0.63 ± 0.03
DNA mass (pg.)	3.42 ± 0.09	3.30 ± 0.10	3.34 ± 0.09	3.36 ± 0.09	3.23 ± 0.09
Total protamine mass (pg.)	2.90 ± 0.12	2.85 ± 0.14	2.98 ± 0.13	2.87 ± 0.15	2.36 ± 0.12
Total protamine mass/DNA mass	0.85 ± 0.05	0.86 ± 0.05	0.89 ± 0.06	0.85 ± 0.06	0.73 ± 0.04

[a]Derived values of DNA and total protamine mass in each species as well as ratio of total protamine mass to DNA mass are also shown. Each mass and ratio measurement is expressed as a sample mean ± sample standard deviation of the individual single sperm nuclei values.

portion of protamine 1 and 2 present in the sperm of these species. Our analyses of pooled stallion sperm in this study revealed that 13.8 ± 0.7% of the protamine bound to DNA in the sperm's nucleus is protamine 2.

Figure 3 shows the ratio of total protamine mass to DNA mass in the five species of sperm examined. This mass ratio is similar for bull, stallion, hamster, and mouse sperm. For the hamster and bull data (which display the largest difference for the four species) students t-tests reveal there is no significant difference between the total protamine to DNA mass ratios for the two species at significance levels of 0.03. Human sperm, however, contained ~18% less protamine inside their nuclei than sperm obtained from the other four species. In human sperm this results in a protamine to DNA ratio that is ~15% lower than those for the other four species examined. Performing a students t-test on the protamine to DNA mass ratio distribution for human sperm with that of each of the other four species yields a significance level of less than 0.001 in each instance. This indicates that the protamine to DNA mass ratio is significantly different in human sperm.

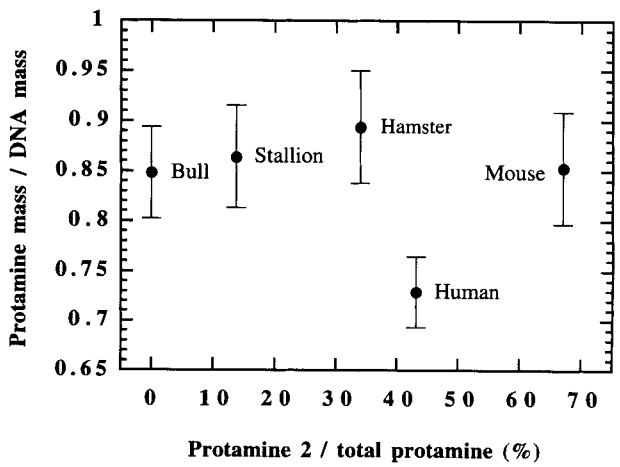

FIG. 3. Scatter plot of total protamine mass/DNA mass in amembraneous sperm nuclei vs. protamine 2/total protamine content. The data displayed in this plot are tabulated in Table 2.

DISCUSSION

The DNA values we obtained by analyzing individual cells in this study are similar to the DNA contents determined previously for bull and mouse sperm by inorganic phosphorus analysis, deoxyribose analysis, and absorption spectroscopy at 260 nm using pools of sperm (29) or by cytomorphometry and cytospectrophotometry of individual sperm (29,32). Those studies found DNA masses

in bull and mouse sperm ranged from 3.1 to 3.6 pg/cell, averaging around 3.3 pg/cell. In this study, bull, mouse, hamster, and stallion sperm all appear to contain similar amounts of DNA ~ 3.3 to 3.4 pg/cell. The lower average DNA content we obtained for the human sperm nucleus is close to the 3.14 pg determined by White et al. (40), but substantially higher than the 2.46 pg obtained by Leuchtenberger et al. (23) and 2.66 pg determined by Ackerman and Sod-Moriah (1). Values for hamster and stallion sperm have not, to our knowledge, been reported previously.

The value obtained by PIXE for the protamine content of the mouse sperm nucleus (2.87 ± 0.15 pg) is also similar to the total protein measurements determined previously by quantitative amino acid analysis, absorption spectroscopy at 230 nm, and ultracentrifugation in cesium chloride gradients using pooled mouse sperm. Whereas these earlier analyses indicated sperm contained ~ 3.0 pg protamine per cell, the actual values varied from 2.8 to 3.2 pg (29). Similar measurements have not been made using bull, hamster, stallion, or human sperm.

Because bull sperm contain only protamine 1, the values determined in this study for DNA mass per sperm head and total protamine mass per sperm head can be used to calculate the length of DNA (number of base pairs) available for binding to a single protamine 1 molecule in bull sperm. The result, 11.4 ± 0.7 bp, fits well with computer modeling studies of the complex that suggested ~10 bp would be required for binding the arginine-rich DNA binding domain of protamine 1 (20). If we assume that complete charge neutralization occurs upon protamine binding, then all 22 arginine residues located in the central DNA binding domain would be required to form salt bridges to phosphate groups in the phosphodiester backbone of DNA.

The results obtained for stallion, hamster, and mouse sperm provide additional information about how protamine 1 and 2 contribute to the packaging of DNA in the sperm of species that contain both protamines. Our measurements demonstrate that the total protamine content of the sperm nucleus, relative to mass of DNA present in the head, is similar for bull, stallion, hamster, and mouse. However, the sperm of these four species contain marked differences in their protamine compositions. Bull sperm have been shown to contain only protamine 1 (16,26), whereas stallion (2,10), hamster (17), human (7,30,35), and mouse (8,13,29) sperm have been demonstrated to contain both protamine 1 and protamine 2. In addition, stallion, hamster, human, and mouse sperm have been determined to contain very different amounts of protamine 2. Our analysis of stallion sperm revealed that ~ 14% of the protamine present in the sperm is protamine 2. Previous studies have determined that protamine 2 constitutes 34%, 43%, and 67% of the protamine present in hamster, human, and mouse sperm, respectively. The data obtained for the total protamine contents of bull, stallion, hamster, and mouse sperm indicate that as the protamine 2 content of the sperm chromatin increases in these species, the protamine 1 content must decrease proportionately. This implies that every turn of DNA in mouse sperm, as one example, cannot be complexed with a protamine 1 molecule as it is in bull sperm.

If we assume that the length of DNA required for protamine 1 binding is identical in bull and mouse sperm, the data provided by this study allow us to estimate the length of DNA occupied by a single protamine 2 molecule. Using the data we obtained for mouse sperm, our calculations indicate that each mouse protamine 2 molecule would have 15.2 bp of DNA available for binding—suggesting that each protamine 2 molecule may cover a slightly longer strength of DNA (33% more). Similar results were obtained using the stallion and hamster data. If each phosphate group in DNA bound to protamine 2 forms a salt bridge to either an arginine or lysine residue in protamine 2, these results suggest that the entire length of the protamine 2 molecule must be bound to DNA. A binding site of 15 bp would require 30 arginine or lysine residues, and mouse protamine 2 contains only 32 arginine and 3 lysine residues distributed throughout its length.

One apparent discrepancy is the mass of protamine we observed in human sperm; it is significantly lower than that determined for the other four species. This is consistent, however, with the unusual protein composition of the human sperm nucleus. Other studies have suggested that up to 15% of the DNA in the sperm nucleus remains packaged by histones (6,35). The studies that provided this information were conducted using pools of cells, conditions that preclude the investigators confirming the existence of histones as a normal complement of every normal human sperm cell. Since histones do not contain cysteine (sulfur), their presence would appear as an apparent decrease in protamine content in the PIXE measurements of the amembraneous human sperm nuclei. The observed difference in protamine content is almost exactly that expected for a cell that has 15% of its DNA packaged by histones.

The marked insolubility of sperm chromatin and the high affinity of protamine for DNA have often made it difficult to characterize the structure of sperm chromatin using the biochemical and physical techniques that have been applied previously to somatic chromatin and other DNA-protein complexes. Further, sperm chromatin analyses have most frequently been performed on whole semen or pools of millions of cells. In these earlier studies, the investigators could not rule out the possibility that the observed result might reflect the presence of a subpopulation of abnormal sperm or spermatids arrested at a particular point in their development.

We have demonstrated in this study that nuclear microscopy utilizing PIXE can be used to measure bound P and S concentrations in the amembraneous nuclei of individual sperm. The technique is absolute, accurate, and fully quantitative. From these measurements, it is possible to derive the total protamine and DNA content of the sperm nucleus in many instances. Further, as X-rays from all elements are detected simultaneously during PIXE, it

is possible to analyze the same nucleus for other bound elements, such as trace metals, down to part per million concentrations. For the work presented here, exposures generally were not long enough to obtain meaningful x-ray counting statistics on bound metals, and so these data have not been reported.

Under the ion beam analysis, conditions used in this study of amembraneous nuclei x-ray yields of phosphorus and sulfur per unit dose remained constant as a function of dose throughout irradiation. In addition, repeat irradiations of several amembraneous nuclei revealed that the spatial distribution of x-rays from phosphorus and sulfur reproduced within counting statistics. These findings indicate that no detectable mass losses occur for phosphorus and sulfur and that the spatial distribution of phosphorus and sulfur within an amembraneous nucleus is not affected at the micrometer scale by the ion beam irradiation conditions. Optical examination of the irradiated area after analysis reveals no observable specimen deformation at the micrometer scale apart from discoloration of the irradiated region. Specimen discoloration is commonly observed when organic specimens are subjected to charged particle irradiation (39) and is often attributed to loss of hydrogen resulting from broken molecular bonds and the formation of color centers within the sample.

In addition to providing information about the structure of sperm chromatin in this study, the demonstrated sensitivity of the PIXE technique should also facilitate the detection of small ($<10\%$) differences in protamine content of individual sperm nuclei in a variety of other studies, including analyses of the nuclear protein compositional changes that relate to male infertility. It is also possible to image an amembraneous nucleus either prior to or after PIXE analysis by a complementary, MeV energy ion beam technique called Scanning Transmission Ion Microscopy, (STIM) (12,34), which is capable of submicrometer scale spatial resolution. This technique provides a projected density profile of the sample, which is useful for morphology assessment (area, shape, and mass). The combination of PIXE and STIM provides a powerful analysis capability to assist in the determination of single cell elemental structure.

CONCLUSIONS

In this study the total protamine mass (protamine 1 plus protamine 2) and the DNA mass have been determined for chromatin inside the nuclei of individual bull, stallion, hamster, human, and mouse sperm from fertile subjects. The results reveal that although the relative proportion of protamine 1 to protamine 2 is highly variable between the species examined, the total protamine mass to DNA mass ratio is similar for bull, stallion, hamster, and mouse sperm nuclei. Using this information, the length of DNA that could be covered by a single protamine 1 molecule in bull sperm has been calculated to be 11.4 ± 0.7 bp. Similar calculations using the data we obtained for stallion, hamster, and mouse sperm suggest that protamine 2 may bind to a slightly longer stretch of DNA (~15 bp). In contrast, the PIXE data obtained for human sperm nuclei indicate human sperm contain significantly less protamine. This observation is consistent with other studies, which suggest that as much as 15% of the DNA in human sperm remain packaged by histones. The DNA and total protamine masses have been derived from nuclear microscopy measurements in which PIXE has been used to determine the amount of phosphorous and sulfur inside the nuclei of individual sperm. The technique is absolute, accurate, and fully quantitative. The technique shows promise of being able to resolve theories regarding the importance of protamines to male infertility, to identify biochemical defects responsible for certain types of male infertility, and to perform quantitative measurements needed to identify certain features of sperm chromatin structure.

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